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Milk Nitrogenous Components as Measured by Near Infrared Spectroscopy-study on the Repeatability for Predicting Different Population Samples

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Abstract The study on the use of near infrared reflectance spectroscopy (NIRS) analysis for measuring milk nitrogenous components (crude protein, true protein, casein and milk urea nitrogen (MUN)) was carried out using one hundred seventy milk samples collected from lactating Holstein cows used in three feeding experiments. Experiment 1 consisted of ninety-six milk samples using sixteen mid-lactation cows fed a basal ration containing corn silage, Italian ryegrass wafer, Alfalfa haycube, corn flake and commercial concentrate with four kinds of supplementation, (1) corn gluten meal (CGM), (2) fish meal (FM), (3) roasted soybean meal (RSBM), and (4) defatted soybean meal (SBM) for each four animals. Experiment 2 consisted of forty-two milk samples from seven early lactation cows raised under total mixed ration (TMR) feeding management containing corn silage, Timothy hay and concentrate. Experiment 3 consisted of thirty-two milk samples from eight early lactation cows raised under two rations feeding management, (1) basal ration supplemented with SBM, and (2) basal ration supplemented with mixed of SBM and FM. Basal ration in this experiment was similar with that given in experiment 1. The samples from experiment 1 and 2 were randomly separated into two groups, (1) calibration set samples (n=84), (2) validation set samples (n=54), while the milk samples from experiment 3 were grouped as (3) application set samples. These groups were used for developing calibration equations, validating the equations, and for evaluating the reproducibility when the calibration equations were employed to samples from different population. Using four combinations of wavelengths at 1650, 1698, 1738 and 1756 nm, the accuracy and reproducibility of measurements were high for crude protein, true protein and casein, but not for MUN.

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Key words : NIRS, Milk nitrogen components, MUN

Focus on the determination method of protein has been on the increase because the impact of protein fraction in milk and the trend of interest in the protein content of milk concerns human health. Protein in milk called total protein (CP) is conventionally determined by multiplying with 6.38 to the total nitrogen compound obtained from Kjeldahl method, or practically by Milkoscan. There are two main broad fractions of protein in milk, (1) true protein (TP) (such as casein, serum albumin) and (2) non protein nitrogen (NPN) which has a different availability for human food. NPN in milk is mainly composed of urea N

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accounting for 20 to 75% of total NPN[5] which is quite variable with cow, breed, stage of lactation and season, and diet[3]. As to feed, the degradability of feed protein in rumen is positively correlated with blood urea nitrogen (BUN) which is the primary source of milk urea. Although milk urea nitrogen (MUN) is not available for food, it is very useful as indicator of nutritional status of the cow and as an index of the adequacy of protein to energy ratio of the diet at the herd level[8,9] and the efficiency of protein utilization[12].

Measurement of these nitrogenous components in milk is valuable to maintain the animal performance and for milk quality. However, the conventional analysis method for these components is time consuming and cannot fulfill the need of fast information and routine management. The practical rapid measurement using Milkoscan does not provide the true protein and casein measurements. Another rapid and accurate method using near infrared spectroscopy (NIRS) technology may fulfill the need. This basic study on the determination of nitrogenous components of milk was done to develope a continuous system to monitor the nutritional status and to evaluate milk quality using NIRS technology.

Materials and Methods

Milk samples

A total of 170 milk samples collected from three feeding experiments using thirty one Holstein cows raised in experimental barn were used in this study. Animals were fed the rations formulated to fulfill the maintenance and production requirements level[1]. The experiments were conducted as follows :

Experiment 1. Ninety-six milk samples of composites of evening and morning milk collected from feeding trials using sixteen cows in mid-lactation (avg BW : 585 kg) fed a basal ration containing corn silage, Italian ryegrass, Alfalfa haycube, corn flake and commercial concentrate were used. Four proportions and types of crude protein (CP) sources were supplied in the ration. Each group of four cows received the supplemented ration as follow, (1) corn gluten meal (CGM, provided 26% total of CP), (2) fish meal (FM, 26% of total CP), (3) roasted soybean meal (RSBM, 26% of total CP), and (4) defatted soybean meal (SBM, 28% of total CP).

Experiment 2. Forty-two milk samples of evening and morning milk were collected from seven cows in early lactation (avg BW : 552 kg) raised under total mixed ration (TMR) feeding management. The TMR given in this experiment consisted of corn silage, Timothy hay and concentrate to make 15% CP ration. Milk samples from cows given these rations were collected on week-4 after parturition for three consecutive days.

Experiment 3. Thirty-two samples of evening and morning milk were collected from eight cows in early lactation (avg BW : 576 kg) raised under two rations feeding management. The rations given in this experiment were (1) basal ration supplemented with soybean meal provided 48% of total CP, and (2) basal ration supplemented with mixture of soybean (20% of total CP) and fish meal (25% of total CP). Basal ration in this experiment was similar with that given in experiment 1. The rations were fed to each four cows. Milk samples were collected on week-4 after parturition for two days.

The rations intake in three experiments are presented in Table 1. Metabolizable energy value was calculated using the value in Japanese Feeding Standard for dairy cattle[1]. Milk was analyzed for total crude protein (CP), true protein (TP), casein and milk urea nitrogen (MUN). Nitrogen content was determined by Kjeldahl N method. True protein and casein were precipitated by 24% TCA treatment and 0.1 N HCl treatment[6], respectively, prior to N determination. Milk urea nitrogen was determined by Urease Indophenol method using a commercial kit analysis (Wako pure chemical Ltd., Japan).

Measurements of NIR spectra and data analysis

NIR spectra of the milk samples were measured with a Pacific Scientific (Neotec) model 6500 instrument (Perstorp Analytical, Silver Spring, MD). The samples were incubated in a 40℃ waterbath prior to the NIR measurements using a transmittance cell (thickness : 1 mm). The spectral data were analyzed by ISI (InfraSoft International, Port Matilda, PA) software. Measurement was made on the second derivative of log (1/A), where A is absorbance. The

Table 1. Average of daily intake of the diets and milk yield from three feeding trials used in this study

	Exp. 1				Exp. 2	Exp. 3	
Feed supplement	CGM	FM	RSBM	SBM	TMR	SBM	SBM+FM
Daily Intake (kg DM/d)							
DM	20.6	21.0	20.4	20.8	18.6	19.7	21.6
CP	3.1	3.2	3.1	3.1	2.8	3.2	3.5
CP (%DM)	15.0	15.2	15.2	14.9	15.1	16.2	16.2
ME (MJ/kg DM)	10.1	10.9	10.2	10.1	11.1	11.9	11.8
Milk yield (kg/d)	30.7	30.4	32.0	29.0	31.8	36.9	39.3

DM : dry matter, CP : crude protein, ME : metabolizable energy, CGM : corn gluten meal, FM : fish meal, RSBM : roasted soybean meal, SBM : soybean meal, TMR : total mixed ration.

spectra was recorded of the range of 1100-2500 nm and was read at 2 nm interval. However, for developing calibrations equations in this study, the wavelengths were chosen between the range of 1400 and 1770 nm which was reported by consisted of several component bands from various components in milk[16]. Multiple linear regression (MLR) was used in the development of calibration equation with maximum four wavelengths. This is because an available commercial instruments are limited up to four wavelengths combination only. The form of calibration equation was,

$$Y = a + b(X_1) + c(X_2) + d(X_3) + e(X_4),$$

where ;

Y is variable to be predicted commonly obtained by reference method ;

a, b, c, d, and e, are the constants ; and

the X_n is a level of absorbance at "n" wavelength.

In this study, the calibration equations for CP, TP and casein were developed using the same combination of selected wavelengths. This was done with a consideration that proteins in milk (CP, TP, casein) are mainly formed in the same structures, therefore the selected wavelengths should be available for all protein form in milk. However, because of the different levels of CP, TP and casein in milk, adjustment was made on the constants for multiplying the level of absorbance to fit with each component.

Calibration equation development

One hundred thirty eight milk samples collected from experiment 1 and experiment 2 were randomly separated into two groups for NIR spectroscopy analysis. This was done to achieve a representative calibration in further application due to recommendation of Shenk *et al.*[13]. The first group was a set of calibration samples (n=84), which was used for developing the calibration equation. The second group was the validation set samples (n=54), which was used for validating the calibration equation developed from the first set of samples. The predicted values by NIR were then compared with the reference method to examine the accuracy. The reliability of calibration equations prior to further application was established based on the values of correlation coefficients (r), standard error of prediction (SEP) and RPD (the ratio of standard deviation of reference data in validation set samples to SEP). Williams[17] introduced the limit value of RPD adequate for screening as 2.5 or higher, with higher value indicating higher accuracy. The validated calibration equations were then applied to predict the thirty two milk samples from different population collected from experiment 3. This evaluation was done to check the reproducibility of the equations.

Results and Discussion

The range of milk nitrogenous components used in this study for CP, TP, casein and MUN, and the summary of NIRS measurements are presented in Table 2. Four wavelengths at 1650, 1698, 1738 and

Table 2. The range of nitrogenous components in milk samples used for the calibration and validation set samples and their coefficient correlation, standard error and RPD values

	Calibration			Validation			
	range	R	SE	range	r	SE	RPD
Crude protein, %	2.35- 4.07	0.92	0.13	2.50- 4.22	0.92	0.13	3.1
True protein, %	2.19- 3.95	0.92	0.13	2.22- 4.10	0.92	0.13	3.0
Casein, %	1.91- 3.34	0.90	0.11	1.90- 3.46	0.91	0.10	3.1
MUN (mg/100 m*l*)	6.12-18.61	0.26	1.00	7.20-17.80	0.34	1.08	2.5

R, r : coefficient correlation, SE : standard error, RPD (the ratio of standard deviation to the standard error in the prediction samples) values.

1756 nm were selected for developing the calibration equations of CP, TP and casein. The first wavelength appeared in the area of protein at 1640-1680 nm[7], while another wavelengths were assigned to CH_2 bands[7,16].

Coefficient correlation and standard error obtained from the calibration and the prediction samples are presented in Table 2. The correlation coefficient values of CP, TP, and casein in the calibration were fairly high being 0.92, 0.92 and 0.90, respectively, and the SE values of CP, TP, and casein were 0.13, 0.13 and 0.11, respectively. However, a poor correlation coefficient was found in MUN (R=0.26). The use of these calibration equations for determining nitrogenous components in the validation set samples obtained the r and SE values for CP, TP and casein were similarly high with those found in calibration set samples, but were low for MUN. However, the judgement based on RPD found that all equations developed from calibration set samples were higher than or the same with 2.5 indicating that the calibration equations were suitable for further use.

Evaluation of reproducibility

Many studies on NIRS for predicting biological components in feedstuff[14], blood plasma[4] or in milk[11,15] showed the possibility of NIRS to predict the components in their validation set samples only, which are factually generated from the same population. The study on further application on samples from different population was limitedly reported[2,10]. The problem on application for milk samples possibly arise due to the factors affecting the characteristic of samples, such as feeding regimes, genetic, seasons, stage of lactation and lactating period.

Table 3. The range of nitrogenous components in milk samples used for application set samples and its coefficient correlation, standard error and RPD values

	Application				
	Range	Mean	r	SE	RPD
Crude protein, %	3.02- 3.86	3.28	0.94	0.09	2.6
True protein, %	2.68- 3.71	3.07	0.93	0.10	2.8
Casein, %	2.31- 3.11	2.55	0.93	0.08	2.6
MUN (mg/100 m*l*)	4.85-10.40	7.34	0.29	0.66	2.3

r : coefficient correlation, SE : standard error, RPD (the ratio of standard deviation to the standard error in the prediction samples) values.

In this study, examination of reproducibility was carried out the validated calibration equations to predict the different samples collected from experiment 3. The background of feeding management of this set sample was similar with experiment 1, and the background of early lactation cows was similar with experiment 2. The use of milk samples from experiment 3 for reproducibility test is reasonable because the background of these samples were represented in the samples used in developing calibration equations[13]. The results of prediction are presented in Table 3 and figure 1. The r values of CP, TP and casein were high (0.93), while the SE values were low (0.10), respectively, with the RPD values higher than 2.5. The results showed the accuracy and reproducibility of the calibration equations developed using the same combination of four wavelengths for CP, TP and casein. However, the prediction for MUN in

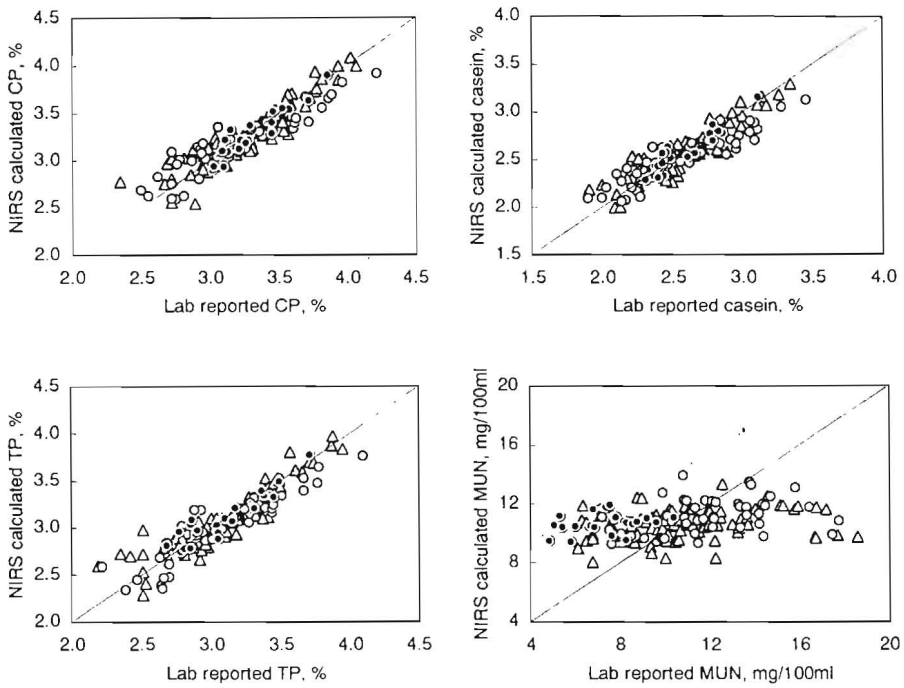

Fig. 1. Plotting the results of crude protein (CP), true protein (TP), casein and urea nitrogen (MUN) components in milk in the calibration (△), validation (○) and application (●) set samples as predicted by NIRS analysis.

application set samples resulted in low coefficient correlation ($r=0.29$) and RPD ($=2.3$) indicated the failure in the measurement.

Our preliminary study on the MUN prediction (unpublish) using various combination of wavelengths of milk spectra and using the selected wavelengths based on the spectra of urea standard solution as a reference also obtained unsuitable results. It was considered because the recorded milk spectra representing the whole information of components in milk included fat, total protein and lactose. The urea concentration in milk exist in trace amounts and therefore is possible to be concealed by other stronger components. For the purpose of strengthening the information of MUN in the spectra used for calculation, the spectra may be better obtained from defatted milk or by the spectra of clear supernatant of precipitated milk samples. Consequently, with this treatment on milk samples the prediction of milk nitrogenous components could not be done at once. However, since MUN is useful for detecting nutritional status in animals, further study is necessary.

In conclusions, the results of this study showed the accuracy and efficient reproducibility of the use of four wavelengths combination at 1650, 1698, 1738 and 1756 nm for CP, TP and casein measurement, but failed to predict MUN. The failure in the prediction of MUN was due to (1) the MUN spectrum was almost concealed by the more dominant components in milk, and (2) the compulsive use of four wavelengths in prediction of CP, TP and casein was not suitable for MUN because MUN is a non-protein N component.

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